Cytotechnology (2005) 49:95–107
DOI 10.1007/s10616-005-4022-6

Optimization of a MRC-5 cell culture process for the production of a smallpox vaccine

Florence Wu[1], Kesav Reddy[1], Isabelle Nadeau[1], John Gilly[1], Sara Terpening[2] and David J. Clanton[2,*]

*[1]BioReliance, Invitrogen Bioservices, 14920 Broschart Road, Rockville, MD 20850; [2]DVC LLC, a CSC company, 64 Thomas Johnson Drive, Frederick, MD 21702; *Author for correspondence (e-mail: dclanton2@csc.com; phone: +301-607-5053; fax: +301-607-5099)*

Received 26 April 2005; accepted in revised form 11 October 2005

Key words: Cell culture, Cell factories, Design of experiments, MRC-5 cells, Optimization, Smallpox vaccine, Vaccinia virus

Abstract

A cell culture process adaptable to produce smallpox vaccine at large scale has been developed. To achieve this, Design of Experiments (DOE) was applied to identify and optimize critical cell culture process parameters for MRC-5 cell growth and recovery during cell expansion. For cell growth, a 2^{5-1} partial factorial (two level, five factor, 16 conditions) study was designed to evaluate the effects of basal media, seeding density, culture volume, feeding frequency and serum concentration on population doubling level (PDL) after 6–7 days in adherent T-flask cultures. Results indicated that lowering the cell density to 1×10^4 cells/cm^2, increasing the culture volume to 0.5 ml/cm^2 and increasing serum concentration to 20% significantly improved cell expansion. These findings correlated with PDLs above 2.0 and cell densities above 1×10^5 cells/cm^2 at the end of the study period. For cell recovery at passaging, a similar DOE was used to evaluate the effect of trypsin concentration, solution temperature, duration of treatment, incubation temperature and duration of standing time between quenching and reseeding. By increasing the trypsin treatment duration to 60 min and lowering the standing time between quenching and reseeding to within 1 h, the recovery of the MRC-5 cells was greatly improved. By using these newly defined conditions, a two-fold improvement in cell expansion was consistently achieved in both roller bottles and 10 layer Nunc® Cell Factories (Cell Factories). Application of these new conditions for current Good Manufacturing Practices (cGMP) production of MRC-5 cell banks and clinical material demonstrated predictably high cell expansion as well as significantly higher production of vaccinia virus, thus providing the basis for manufacturing vaccinia virus at large scale. These findings demonstrate the need for cell culture optimization and the effectiveness of DOE to rapidly define processes suitable for cGMP manufacturing of a smallpox vaccine or other viral vaccine products.

Introduction

Smallpox was declared eradicated by the World Health Organization in 1980 with the last reported case of natural infection occurring in Somalia in 1977 (Fenner et al. 1988), and there is no evidence that smallpox will re-emerge as an endemic disease. Recent world events have made

the re-introduction of a new smallpox vaccine highly desirable. The current threat from smallpox is that this virus may be used as a potential bioterrorist weapon or biological warfare agent (Henderson et al. 1999). Since its eradication, a large percentage of the world's population is now susceptible to smallpox infection and it is feared that, should the smallpox virus be released, the disease would spread rapidly.

The current supply of smallpox vaccine licensed in the United States, Dryvax^{TM}, was produced by methods unacceptable by current regulatory standards. This vaccine is also in limited supply, since production was terminated in the early 1980s. To address this, a MRC-5 cell-culture derived process was developed by using a three-times plaque purified vaccinia virus strain (TSI-GSD-241), developed at the US Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD.

Our recent experiences in expanding MRC-5 cells, a human diploid embryonic lung fibroblast cell line (Jacobs et al. 1970) set aside for vaccine production, showed slow growth and low PDLs of less than 1.5 per culture passage. Based on these observations, cell expansion trains are long in duration, inefficient in yields, and labor intensive, particularly as production scale increases. It was also noted that low cell yields can also result in sub-optimal production of vaccinia virus, resulting in lower than desired virus production yields per lot (unpublished data). These observations motivated the need to explore cell growth and trypsinization parameters to improve cell yields and recovery per passage during cell train expansion and serial propagation.

Many variables affect cell growth and recovery. Due to the large number of variables under consideration, factorial DOE was applied to concurrently study multiple variables while reducing the number of required experiments (Box et al. 1978; Moran et al. 2000; Chun et al. 2003). Each of two areas of investigation was explored separately. Initial studies were conducted in T-flasks to gain broad insight into trends and to identify critical cell culture parameters affecting MRC-5 cell growth and recovery during passaging. These results were then used to select new conditions for further characterization and optimization in roller bottles and Cell Factories. Finally, these new conditions were used to illustrate the applicability of this approach to develop a cGMP clinical manufacturing process of vaccinia virus in tissue culture for a new smallpox vaccine.

Materials and methods

Cell culture and maintenance

MRC-5 cells (originally obtained from the ATCC for vaccine use) from working cell banks (*DVC*) were thawed at passage 31 and cultured for the first 1–2 weeks in 75 cm^2 (T-75) and 225 cm^2 (T-225) tissue culture T-flasks (*Costar*). All studies were completed several passages before cells entered senescence (data not shown). An MRC-5 cell train was maintained in T-225 flasks in 'original' medium Modified Eagle's Medium (MEM; *Life Technologies, Inc.*) supplemented with 1 mM sodium pyruvate (NaPyr, *Biowhittaker*), 1% (v/v: volume of supplement to volume of medium) non-essential amino acids (NEAA, *Biowhittaker*) and 10% (v/v) heat inactivated fetal bovine serum (FBS, *Hyclone*) and used to seed T-75 flasks for each of the factorial design experiments. A number of T-225 flasks were shifted to Dulbecco's Modified Eagle's Medium, F-12 Hams Mixture (DMEM/F12, *Life Technologies, Inc.*) supplemented with 2 mM glutamine, 1 mM NaPyr and 10% FBS to adapt them to the richer medium for one to two passages before the start of the experiments. T-flasks were seeded at 2×10^4 cells/cm^2 and passaged every 3–4 days until start of the experiments. T-flasks and 10-layer Cell Factory cultures were maintained in a humidified incubator at 36 °C and 5% CO_2. After the factorial experiments, unless otherwise specified, cells were rinsed with phosphate buffered saline (PBS), treated with trypsin (*BioWhittaker*) for 5–10 min at room temperature, and the trypsin activity quenched with DMEM (*Life Technologies, Inc.*) supplemented with 2 mM glutamine, 1 mM NaPyr and either 10 or 20% FBS. Cell suspensions were counted by using a hemocytometer, and viability was assessed by the Trypan Blue exclusion method.

Virus strain

TSI-GSD-241 is a three times plaque purified vaccinia virus strain developed by USAMRIID. This seed was used to establish a master and working virus bank, CCSV, characterized for

potency and purity. The working virus bank was used for the studies presented here.

Cell growth factorial studies

A 2^{5-1} factorial DOE was applied to investigate five factors at two levels in 16 conditions. The five factors and the two levels (determined around normal growth conditions) are listed in Table 1, and the 16 conditions representing the forward design are shown in Table 2. A reverse factorial, defined as the set of opposite conditions from the forward factorial, was also conducted to assess reproducibility of results and to complete the full design. An additional condition of triplicate flasks cultured under the original parameters was included as a control to compare with the new conditions. These were also used to calculate the standard error of measurement for each block of experiments.

This study was conducted in T-75 flasks, and all parameters were normalized to surface area for ease of subsequent data analysis. When cells reached confluence or after 6–7 days of culturing, each flask was assessed for cell density, viability and pH. Cell density and viability were assessed by a hemocytometer and the Trypan Blue exclusion method. The pH of spent medium from the T-75 flasks was measured by use of pH probe (*Mettler Toledo*). The PDL was calculated [PDL = ln (viable cell density at harvest/viable cell density at seeding)/ln (2)] and served as the output variable.

Cell recovery factorial studies

A 2^{5-1} factorial DOE was applied to investigate five factors that might affect recovery of viable cells upon harvest/transfer. These factors were evaluated at two levels in 16 conditions. The five factors and the two levels are listed in Table 3 along with normal growth conditions, and the 16 conditions representing the forward design are shown in Table 4. A reverse factorial, defined as the set of opposite conditions from the forward factorial, was also conducted to assess reproducibility of results and to complete the full design. An additional condition of triplicate flasks cultured under the original parameters was included as a control to compare against the new conditions. These were also used to calculate the standard error of measurement for each block of experiments.

This study was conducted in T-75 flasks, and all parameters were normalized to either surface area or cell number for ease of subsequent data analysis. After 6–7 days of culturing, each flask was trypsinized and assessed for cell density. Ability of recovered cells from each of the different conditions to re-attach and grow in a subsequent passage was also assessed. Cell yields for each condition were normalized to the cell yield in control flasks treated under the original recovery conditions (see Table 4). These normalized values served as the output variable for the factorial design analysis.

Table 1. Variables for Cell growth factorial design[a].

Variable	Low range	High range
A Basal media	MEM	DMEM/F-12
B Seeding density	1×10^4 cells/cm^2	4×10^4 cells/cm^2
C Culture volume	0.15 ml/cm^2	0.5 ml/cm^2
D Feeding frequency	None	Once per 3–4 days
E Serum concentration	5%	20%

[a]Control (original) conditions are shown in Table 5.

Factorial design analysis

Full factorial design analysis

The responses or yields are examined in the normal probability plot of effects against the cumulative probability function (using the software package: Minitab™ Statistical Software v13). Random effects and interactions cluster along a line on the normal probability plot. Significant effects and interactions lie outside the line of randomness either on the positive or negative effect axis. A positive effect on yield due to a variable indicates that an increase in the levels of the variable would lead to high yields and similarly decreasing the levels of the variable would lead to low yields. A negative effect on the yield due to a variable indicates increasing the level of that variable would lead to low yields and conversely, decreasing the level of the variable leads to high yields. A positive effect on the yield due to interaction of two variables indicates increasing or decreasing both variables concurrently would have a positive effect on the yield. A negative effect on the yield, due to the interaction of two variables, indicates that increasing one variable at the

Table 2. Summary of conditions and results for MRC-5 cell growth factorial.

Condition	Summary of conditions					Cell count $\times 10^4$/cm^2	PDL	pH
1	M	1	L	N	20	2.90	1.54	7.10
2	M	1	L	Y	5	2.36	1.24	7.26
3	M	1	H	N	5	2.53	1.34	7.36
4[a, b]	M	1	H	Y	20	4.60	2.20	7.35
5	M	4	L	N	5	3.77	−0.09	7.20
6	M	4	L	Y	20	5.09	0.35	7.20
7[b]	M	4	H	N	20	7.84	0.97	7.23
8	M	4	H	Y	5	3.64	−0.14	7.45
9	F	1	L	N	5	1.42	0.51	6.91
10[a, b]	F	1	L	Y	20	4.12	2.04	6.89
11[a, b]	F	1	H	N	20	4.70	2.23	7.07
12	F	1	H	Y	5	3.60	1.85	7.11
13	F	4	L	N	20	2.54	−0.66	6.70
14	F	4	L	Y	5	3.81	−0.07	6.95
15	F	4	H	N	5	3.60	−0.15	7.05
16[b]	F	4	H	Y	20	9.57	1.26	6.96

[a]Indicates conditions with a PDL >2.
[b]Indicates conditions with cell densities $>4\times10^4$ cells/cm^2.
Basal media – MEM (M), DMEM/F-12 (F); Seeding density – 1×10^4 cells/cm^2 (1), 4×10^4 cells/cm^2 (4); Culture volume – 0.15 ml/cm^2 (L), 0.5 ml/cm^2 (H); Feeding frequency – None (N), Once per 3–4 days (Y); Serum concentration – 5% (5), 20% (20).

expense of the other would have a positive effect on the yield. Further explanation of factorial design of experiments can be found in the reference (Box et al. 1978).

Fractional factorial design analysis

The responses or yields (such as PDLs, cell density recovered after trypsinization) are examined in a normal probability plot of effects against the cumulative probability function (using the software package: Minitab Statistical Software v13). Effects and interactions that are not significant fall random along a line on the normal probability plot. Significant effects and interactions do not lie along the line on the normal probability plot and fall on either side of the line on the positive side or negative side of the *x*-axis. A positive effect on yield due to a variable indicates that an increase in the levels of the variables would lead to high yields and similarly decreasing the levels of the variables would lead to low yields. A negative effect on the yield due to a variable indicates increasing the level of that variable would lead to low yields and conversely, decreasing the level of the variable leads to high yields.

A positive interaction of two variables indicates increasing or decreasing both variables at the same time would lead to an increase in the yield. A negative interaction of two variables indicates that increasing one variable while decreasing or not increasing the other variable would lead to an increase in the yield.

Further explanation of fractional factorial design of experiments can be found in the reference (Box et al. 1978).

Table 3. Variables for MRC-5 cell recovery factorial design[a].

Variable		Low range	High range
A	Time at room temperature post-trypsinization and pre-seeding	1 h	4 h
B	Trypsin incubation time	10 min	60 min
C	Trypsin temperature	4 °C	20 °C
D	Trypsin concentration	0.025%	0.05%
E	Incubation temperature	20 °C	37 °C

[a]Control (original) conditions are shown in Table 6.

Roller bottle scale-up studies

After data analysis of the cell growth and cell recovery factorial studies in T-flasks, conditions were chosen for further study in roller bottles. Roller bottles (*Corning*) were maintained at 36 °C, 5% CO_2 and 0.5 rpm. The set of culture parameters used for these studies is summarized in Tables 5 and 6. For these studies, 1–2 roller

Table 4. Summary of conditions and results for MRC-5 cell recovery factorial.

Condition	Summary of conditions					Cell count $\times 10^4$/cm^2	Cell count normalized[b]
1	4	60	20	H	20	4.58	0.47
2	4	60	20	L	37	7.90	0.82
3	4	60	4	H	37	6.40	0.66
4	4	60	4	L	20	2.53	0.26
5	4	10	20	H	37	3.90	0.40
6	4	10	20	L	20	3.90	0.40
7	4	10	4	H	20	1.70	0.18
8	4	10	4	L	37	2.16	0.22
9	1	60	20	H	37	7.25	0.75
10[a]	1	60	20	L	20	15.00	1.55
11[a]	1	60	4	H	20	15.00	1.55
12[a]	1	60	4	L	37	8.80	0.91
13	1	10	20	H	20	5.50	0.57
14	1	10	20	L	37	1.10	0.11
15	1	10	4	H	37	7.90	0.82
16	1	10	4	L	20	7.60	0.79

[a]Indicates conditions with % recovery >90% of control.
[b]Normalized to control flasks.
Time at room temperature post-trypsinization and pre-seeding – 1 h (1), 4 h (4); Trypsin incubation time – 10 min (10), 60 min (60); Trypsin temperature – 4 °C (4), 20 °C (20); Trypsin concentration – 0.025% (L), 0.05% (H); Incubation temperature – 20 °C (20), 37 °C (37).

bottles were assessed at each time point for cell density, percent viability, and pH when cells reached confluence, or after 6–7 days of culturing. The cell growth profile under these new conditions was compared to that cultured under the original non-optimized conditions.

Vaccinia virus production

MRC-5 cells from the working cell bank were expanded in T-flasks until sufficient numbers of cells were obtained to seed either T-225 flasks or 10-layer Cell Factories for evaluation of vaccinia virus production. Cells were cultured in a modified growth medium (see Table 5) and recovered by using a modified recovery protocol (see Table 6). The new growth medium consisted of DMEM supplemented with 20% FBS, 2 mM glutamine and 1 mM NaPyr. After 6–7 days of culture in this new growth medium, control T-flasks seeded under the same conditions as the Cell Factories were assessed for cell density and viability. This number was used to calculate the amount of virus required for infection. Each T-flask or Cell Factory was infected with a virus bank CCSV (*DVC*), derived from the TSI-GSD-241 vaccinia virus strain. Cells were infected at a multiplicity of infection (MOI) of 0.1 PFU/cell in 2 l of infection medium consisting of DMEM supplemented with 1 mM NaPyr

Table 5. Summary of original and new MRC-5 cell growth conditions.

Variable	Original conditions	Optimized
A Basal media	MEM	DMEM
B Seeding density	2×10^4/cm^2	1×10^4 cells/cm^2
C Culture volume	0.18 ml/cm^2	0.3 ml/cm^2
D Feeding frequency	2 × per week	2 × per week
E Serum concentration	10%	20%

Table 6. Summary of original and new MRC-5 cell recovery conditions.

Variable		Original (control)	New (optimized)
A	PBS Rinse volume*	0.02 ml/cm^2	0.02 ml/cm^2
B	Trypsin concentration	0.05%	0.05%
C	Trypsin volume*	0.012 ml/cm^2	0.33 ml/cm^2
D	Trypsin temperature	20 °C	20 °C
E	Trypsin incubation time	5 min	60 min
F	Incubation temperature	20 °C	37 °C
G	Quench volume*	0.02 ml/cm^2	0.06 ml/cm^2
H	Time at room temperature post-trypsinization and pre-seeding	30 min	1 h

*Indicates variables studied separately from factorial design study (data not shown).

and 5% FBS. T-flasks and Cell Factories were maintained in a humidified incubator at 36 °C and 5% CO_2. At 72 h post-infection, Cell Factories were inspected for greater than 80% cytopathic effect (CPE), rinsed with PBS, and then harvested after treatment with trypsin. Recovered cells were resuspended at 4×10^7 cells/ml in a conical tube, and then sonicated to disrupt cells and release virus. Samples post-disruption were taken for virus titration to determine extent of virus production. For virus production studies in T-flasks, the cells were harvested between 48 and 72 h post-infection. For both Cell Factory and T-flask studies, vaccinia virus production was accomplished at early cell passages (between passages 30 and 40), or within 15 passage doubling levels from point of cell thaw from the working cell bank.

Vaccinia virus titration

Vaccinia potency (infectivity) determinations were carried out by plaque assay on VERO cells. VERO 76 cells (*ATTC*) were plated in 6-well tissue culture dishes for 24 h prior to inoculation and were approximately 85% confluent at the time of inoculation. Serial 10-fold dilutions of the test article and positive control virus were prepared in diluent: Eagle's MEM (EMEM) with 1% (v/v) NEAA, 1.5% (v/v) L-glutamine, 10% (v/v) heat inactivated FBS and 0.1% neomycin. After removal of the culture medium from wells of VERO 76 cells, appropriate test article and positive control dilutions, in 0.2 ml volumes, were inoculated in triplicate. An additional six wells were inoculated with diluent to serve as the negative control. Following adsorption for 60 min at 36 °C in a humidified CO_2 incubator, the cells were overlaid with a mixture of 2× overlay medium (2× EMEM supplemented with 4% (v/v) L-glutamine, 1% (v/v) NEAA, 0.1% neomycin, and 1.8% agarose (*SeaKem ME*). The VERO 76 cells were maintained in culture for 48 h after which the cells were overlaid a second time with agarose medium containing the vital dye, Neutral Red.

After an additional 24 h in culture, the unstained areas, or plaques, in all wells containing ≤200 plaques were scored. The number of countable plaques per well were summed and divided by the sum of the product of all relevant dilution factors times the volume inoculated per well to calculate the virus titer in plaque forming units per milliliter (PFU/ml).

Results and discussion

Cell growth optimization

The objective of the cell growth optimization study was to optimize MRC-5 cell growth conditions to achieve greater than 2 PDLs and a viable cell density greater than 4×10^4 cells/cm^2 to enable an economically feasible process. The variables considered critical for MRC-5 cell growth (Table 1) were established based on our experience with propagation of MRC-5 cells and confirmed by a literature survey for MRC-5 cells and other similar fibroblast cell lines. Two basal media containing different levels of nutrients such as amino acids: MEM and DMEM/F12 (Litwin 1970; Namba and Kimoto 1979), a sparse seeding density and higher seeding density (Agostini et al. 1980), low and high culture volumes (Ryan et al. 1975), no feeding or exchanging medium once during cell growth (Agostini et al. 1980) and low and high serum concentrations (Lambert and Pirt 1975) were used in the factorial design. The levels for these variables were chosen such that the low and high levels were at the outlying ends of the range considered practical. This increases the probability of an effect on the yields, but was within practical limits of processing, e.g., a serum concentration higher than 20% was not considered practical for large-scale cell culture. It was assumed that a change in the variable would indicate the direction of the effect on the yield (an increase in serum concentration from 5% to 20% would lead to an increase or decrease in yield such as PDLs). Future studies are warranted to test for non-linearity by response-surface mapping.

PDL is a variable that is relevant, because our goal was to achieve the required number of cumulative population doublings for subsequent scale-up, regardless of growth rate or confluence. That is, the objective was to evaluate whether lowering the seeding density would allow greater fold expansion while reducing cell seeding. Based on the fractional factorial analysis for cell growth (as seen in Figure 1a and in the conditions in Table 2), it was seen that increasing culture volume from 0.15 to 0.5 ml/cm^2 (condition C) and increasing serum content

from 5% to 20% (condition E) showed a positive effect on PDL. Increasing seeding density from 1×10^4 to 4×10^4 cells/cm^2 (condition B) showed a negative effect (or, decreasing seeding density has a positive effect) on PDL. The analysis of full (32 conditions – Figure 1a) and factorial (16 conditions – Figure 1b) resulted in similar positive and negative effects in the cell growth factorial analysis. However, an additional variable, the feeding frequency (condition D) was also seen to have a moderate positive effect in the full factorial analysis (that is, feeding once after seeding of T-flasks as compared to no feeding led to a higher PDL).

The finding that an increase in culture volume (Table 1, variable C) correlated to increased cell yields may be due to one or more of the following reasons. One hypothesis is that a nutrient(s) in the medium is provided at a higher amount and is supporting the cells nutritionally better than at a lower amount (i.e., lower volume of media). Another speculation is that MRC-5 cells proliferate more strongly under a lower oxygen tension (Taylor et al. 1978; Balin et al. 1984) and this might occur as a result of the increased volume increasing the diffusion length for gas exchange thus decreasing oxygen levels at the monolayer in the T-flask (Randers-Eichhorn et al. 1996). Further work using a dynamic gassed system with dissolved oxygen control is planned to prove this hypothesis.

The increase in serum concentration (Table 1, variable E) in the growth medium resulted in an increase in the PDLs. Thus, non-limitation of growth factor(s) due to the increased serum possibly accounts for increased MRC-5 yields (Lambert and Pirt 1975).

The effect of lower seeding density (Table 1, variable B) on increased PDL can be explained by the contact inhibition effect seen in fibroblast cultures such that cell–cell contact at higher cell densities restricts further growth once confluence has been achieved (Agostini et al. 1980; Wieser and Oesch 1986; Pani et al. 2000).

A moderate positive effect of increased frequency of feeding (Table 1, variable D) was seen in the full factorial analysis of the PDLs (Figure 1a, not seen in the fractional factorial analysis Figure 1b) possibly due to nutrient supplementation (Agostini et al. 1980). A moderate positive effect of increased frequency of feeding on pH (Table 2) was also seen (normal probability plot not shown).

No effect of the basal medium (Table 1, variable A) was seen. Using MEM (less rich relative to DMEM) or DMEM/F12 (rich relative to MEM) as growth medium has no significant effect on the PDLs. However, the use of DMEM/F12 had a negative effect with respect to the pH as analyzed by DOE (normal probability chart is not shown; this effect is also seen in Table 2). The elimination of the pH effect was particularly critical for this program as the design of the vaccine manufacturing process necessitated the need to use pH-uncontrolled system. In an environmentally controlled system like a stirred tank bioreactor (Aunins et al. 2003), this need may have been overcome by controlling for pH via gas mixing. Therefore it was decided to use DMEM (more rich relative to MEM, less rich relative to DMEM/F12) instead of MEM or DMEM/F12 (Agostini et al. 1980). The optimized growth conditions are shown in Table 5.

Cell recovery optimization

The objective of this study was to optimize MRC-5 cell recovery trypsinization conditions to achieve percent recovery greater than 90% of control values. Trypsin temperature, trypsin incubation temperature, trypsin incubation time and trypsin concentration were all considered to be critical for the action of trypsin on the cells. The cells harvested would also be affected by the time spent at room temperature post-trypsinization and pre-seeding due to the extended processing time during large-scale operations. Similarly, it was expected that interaction effects would be seen between the different variables, e.g., between the concentration of trypsin used and the trypsin incubation time and the time post-trypsinization and time pre-seeding. As in the cell growth studies, the levels for these variables were chosen such that the low and high levels were at the outlying ends of the range considered practical. This increased the probability of an effect on the yields, but was within practical limits of processing e.g., the levels for incubation temperature were chosen as close to room temperature for low level or at 37 °C for the high level, an incubator temperature commonly used in cell culture. It was assumed that a change in the variable would indicate the direction of the effect on the yield. Future studies are warranted to test for non-linearity by response-surface mapping.

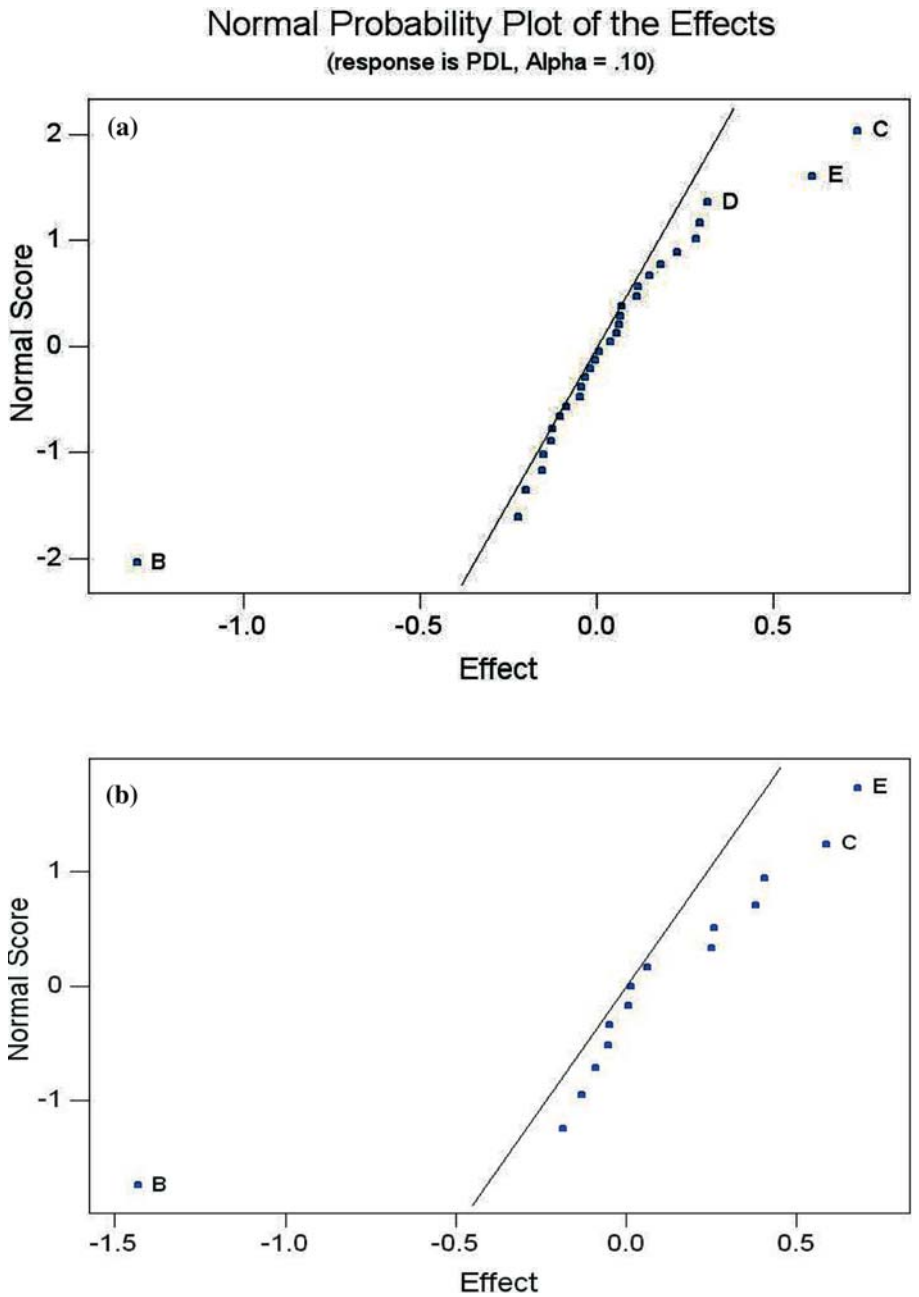

Figure 1. (a) Probability plot for PDL: full factorial cell growth. Significant effects are shown in figure. The higher level of the variables culture volume, feeding frequency and serum concentration show a positive effect, and a lower level of the variable seeding density shows a positive effect on the number of PDLs achieved. (*Significant effect) (A) Basal media. (B) Seeding density.* (C) Culture volume.* (D) Feeding frequency.* (E) Serum concentration.* (b) Probability plot for PDL: fractional factorial cell growth. Significant effects are shown in figure. The higher level of the variables culture volume and serum concentration show a positive effect, and a lower level of the variable seeding density shows a positive effect on the number of PDLs achieved. (*Significant effect) (A) Basal media. (B) Seeding density.* (C) Culture volume.* (D) Feeding frequency.* (E) Serum concentration.*

Based on the full factorial analysis for cell recovery as seen in Figure 2a, it was seen that increasing trypsin treatment duration (condition B) from 10 to 60 min showed a positive effect on cell recovery. Increasing trypsin concentration or increasing the temperature of trypsin and

increasing incubation temperature had no effect on the cell recovery. Increasing time spent at room temperature post-trypsinization and pre-seeding (condition A) from 1 to 4 h showed a negative effect (or, decreasing time temperature post-trypsinization and before re-seeding has a positive effect) on cell recovery.

The analysis of fractional factorial (16 conditions – Figure 2b) and the analysis of the full factorial (all 32 conditions Figure 2a) resulted in similar positive and negative effects in the cell recovery factorial analysis. The positive effect of increased trypsinization time (condition B) is advantageous during processing of multiple flasks during scale-up. It is also particularly advantageous for large-scale operations to perform the trypsinization operation at room temperature (condition E), since there is no effect seen due to the temperature of the trypsin and the incubation temperature. By the same token, it is important to arrange processing logistics to shorten the time that the cells spend at room temperature post-trypsinization and before seeding for further expansion. The optimized cell recovery conditions are shown in Table 6.

Scale-up studies

Results obtained in the factorial studies were applied towards defining a set of optimized cell growth conditions (Table 5) and cell recovery conditions (Table 6) for scale up studies. The new conditions were tested in roller bottles to evaluate applicability for subsequent manufacturing. As seen in Figure 3, MRC-5 cells cultured in the optimized conditions had improved cell growth characteristics as seen by an 8-fold increase in cell density within 5 days of culturing and a higher maximum cell number, compared to only a 2-fold increase in cell number within 7 days of culturing and a lower maximum cell number in the unoptimized control conditions. These new conditions were applied towards a cGMP vaccinia virus manufacturing process, which required expansion of cells in T-flasks and production of virus in 10-layer Cell Factories. A minimum of 12 PDLs was required through the entire expansion process, requiring four MRC-5 sub passages. As seen in Figure 4, the cell growth of MRC-5 cells under the new expansion scheme was highly reproducible over several replicate runs. In each run, the same conditions were used for passaging, and the completion of the runs spanned a period of approximately 12 months.

Cells from the same Working Cell Bank were used to produce vaccinia virus in 10-layer Cell Factories. As seen in Figure 5, original conditions that were used produced an average virus titer of 21.6 ± 10.2 PFU/cell. Upon optimization of growth conditions and switch to the 10-layer Cell Factories, productivity increased to 55.8 ± 20.9 PFU/cell representing a significant savings in time and cost. The difference in productivity was found to be significant (p value $= 0.006$). These findings suggest that optimization of growth conditions for MRC-5 cells resulted in enhanced vaccinia virus production. In essence, this enhancement reduced the number of manufacturing runs by two-thirds.

The scale-up studies demonstrate that the findings from the factorials, which were conducted in T-flasks, are applicable to both roller bottles and larger flat stock systems such as the stacked plate system (Cell Factory). Scale-up from T-flasks into Cell Factories is not always linear, and cell growth performance in T-flasks is generally not worse than in Cell Factories. Therefore, the increase in virus production after culture optimization and scale up is likely due to the change in culture conditions, rather than the change from T-flasks to Cell Factories.

Statistical DOE

The first half-fraction of the factorial design for the cell growth and cell recovery was executed and the results were analyzed. Since we saw a significant effect on the yield due to these variables and the two levels of the variables, the complementary half fraction of the factorial design was implemented to achieve greater redundancy and precision. When the effects and interactions from the fractional factorial design and the complete factorial design for the cell growth and cell recovery study are compared, similar results are seen (Figure 1a and b for cell growth; see Figure 2a and b for cell recovery).

It is therefore useful to consider running a half-fraction factorial DOE and analyze these results. This enables a decision to either proceed to complete

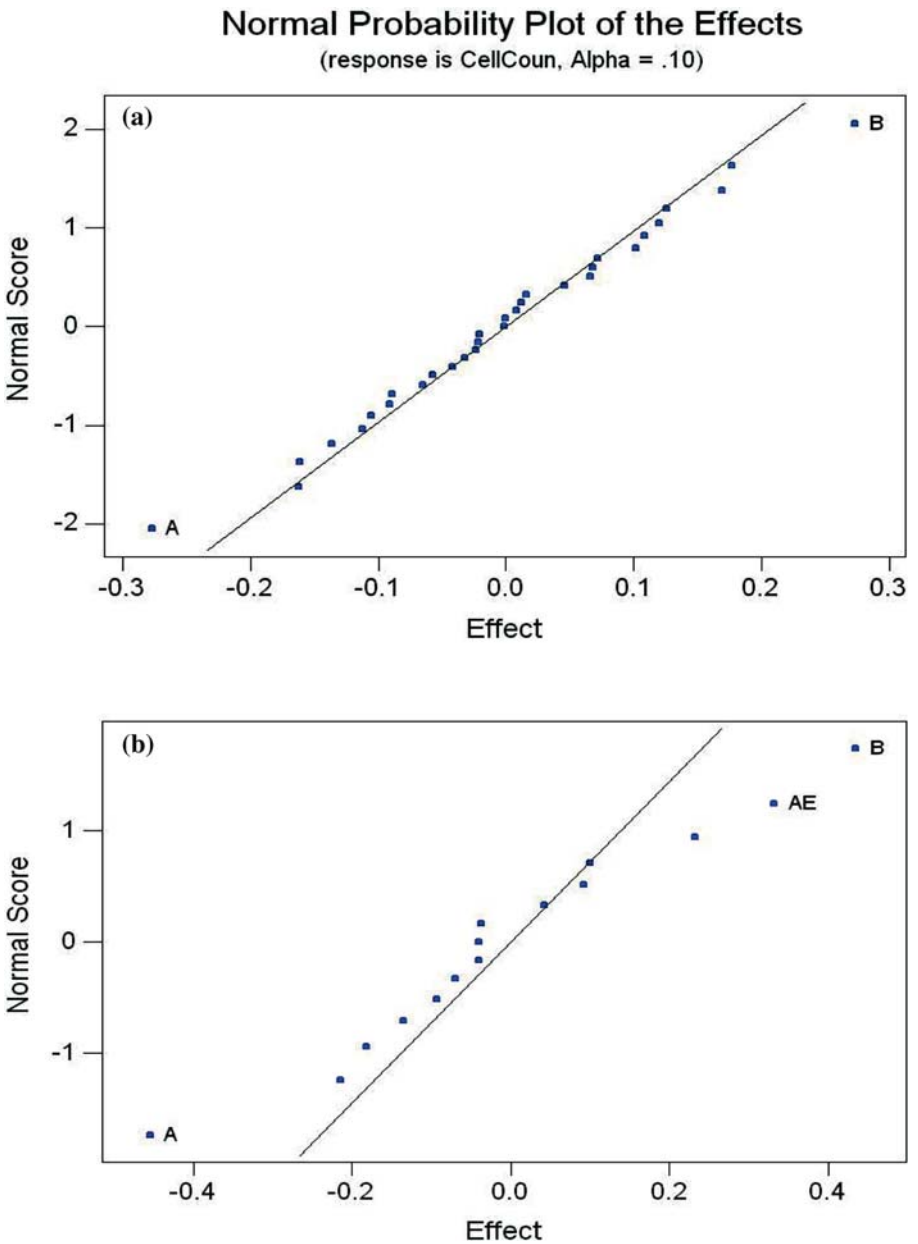

Figure 2. (a) Probability plot for normalized cell number full factorial cell recovery significant effects are shown in figure. The higher level of the variables trypsin treatment duration and time spent at room temperature post-trypsinization and pre-seeding show a positive effect on the normalized cell number recovered (normalized to cell numbers recovered in control flasks). (*Significant effect) (A) Time at room temperature post-trypsinization and pre-seeding.* (B) Trypsin incubation time.* (C) Trypsin temperature. (D) Trypsin concentration. (E) Incubation temperature. (b) Probability plot for normalized cell number fractional factorial cell recovery. Significant effects are shown in figure. The higher level of the variables time spent at room temperature post-trypsinization and pre-seeding and trypsin treatment duration show a positive effect on the normalized cell number recovered (normalized to cell numbers recovered in control flasks). A positive effect is seen for the interaction of time spent at room temperature post-trypsinization and pre-seeding and incubation temperature. (*Significant effect) (A) Time at room temperature post-trypsinization and pre-seeding.* (B) Trypsin incubation time.* (C) Trypsin temperature. (D) Trypsin concentration. (E) Incubation temperature. (AE) Interaction.*

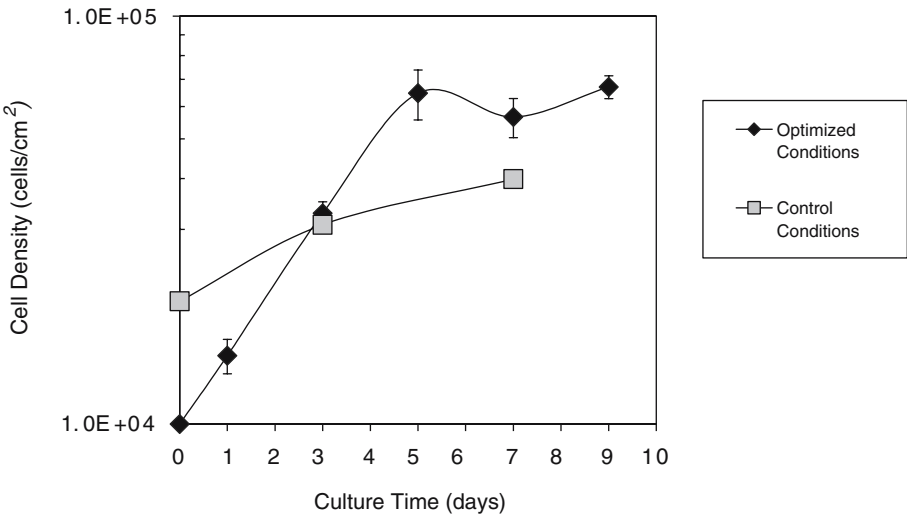

Figure 3. MRC-5 Cell growth in roller bottles. The growth kinetics of MRC-5 cells in roller bottles in optimized and unoptimized conditions are compared.

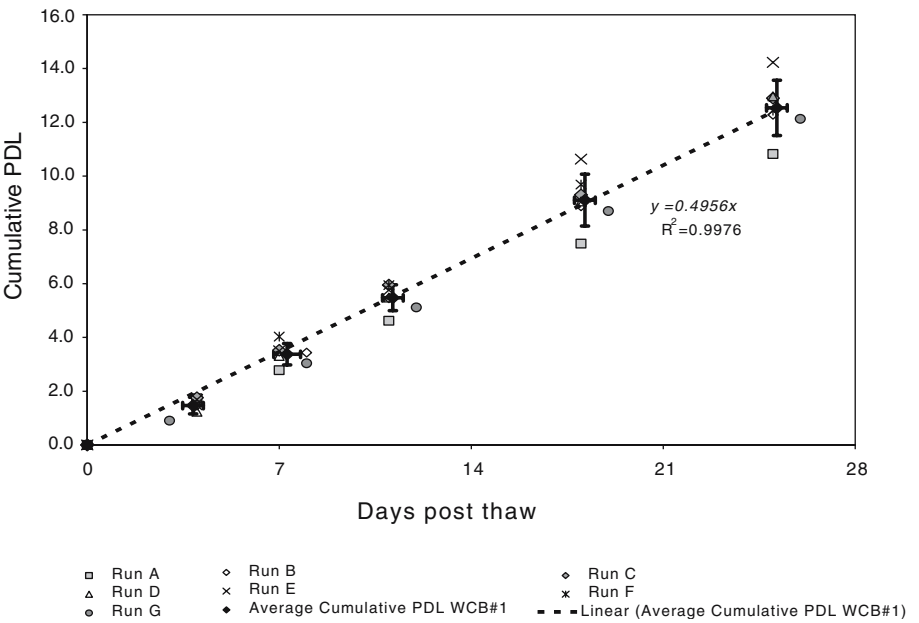

Figure 4. Reproducible MRC-5 cell growth in Nunc® Cell Factories. Reproducible cumulative PDLs were achieved in the cGMP production runs.

the complementary fraction factorial design and analyze the complete set of results or to proceed with an iteration of the fractional factorial that is a modification of the variables or the two levels of existing variables. Further discussion can be found in the reference (Box et al. 1978).

Conclusion

The results presented here show that optimization of MRC-5 cell growth and recovery can be performed using factorial DOE. Optimization conditions of both of these processes are shown in

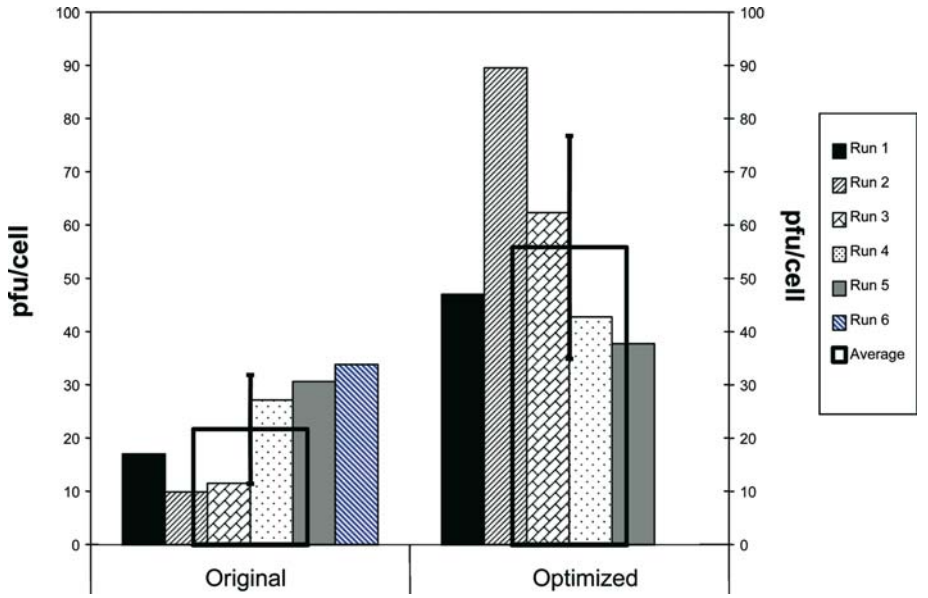

Figure 5. Comparison of vaccinia virus production in original and optimized conditions. The effect of optimized and original growth conditions on vaccinia virus output is compared.

Tables 5 and 6. For cell growth, using DMEM with 20% FBS showed improved growth. Other conditions also were found to affect cell growth. For cell recovery, trypsin incubation had a positive effect, whereas time at room temperature post-trypsinization and pre-seeding had a negative effect. The optimized conditions were used for the large scale manufacturing of vaccinia virus and were shown to significantly improve virus production. Therefore, a manufacturing process for vaccinia virus production in cell culture using a stacked plate system is feasible and practical. Due to optimized cell growth and recovery, 12 PDLs were achieved for cGMP production of the vaccine, essentially a 12-fold doubling was achieved from cell thaw to cell factory (see Figure 4).

The methods presented here demonstrate a practical approach of using Cell Factories for manufacturing of vaccinia virus and other viruses that are replicated in MRC-5 cells. The use of Cell Factories for virus production is particularly advantageous for adherent cells since they are disposable, have few product contact points for a GMP process, are scalable and easily transferable to other manufacturers.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Shantha Kodihalli, Jon Marshall, Jingjin (Jean) Harms, Mary Machan and Sandra Dusing on the Cell Factory studies. The authors also acknowledge the assistance of Don Fine and Robert House for helpful suggestions. This work, funded by the Joint Vaccine Acquisition Program through the Department of Defense (DoD) contract DAMD 17-98-C-8024, does not represent official DoD positions, policies, or decisions.

References

Agostini C.F., Mann G.F., Allison L.M. and Johnson F.H. 1980. Efficiency of medium utilization by human diploid fibroblasts in culture. Dev. Biol. Stand. 46: 51–58.

Aunins J.G., Bader B., Caola A., Griffiths J., Katz M., Licari P., Ram K., Ranucci C.S. and Zhou W. 2003. Fluid mechanics, cell distribution, and environment in CellCube bioreactors. Biotechnol. Prog. 19(1): 2–8.

Balin A.K., Fisher A.J. and Carter D.M. 1984. Oxygen modulates growth of human cells at physiologic partial pressures. Exp. Med. 160(1): 152–166.

Box G.E.P., Hunter W.G. and Hunter J.S. 1978. Statistics for Experiments: An Introduction to Design, Data Analysis, and Model Building. John Wiley & Sons, New York.

Chun C., Heineken K., Szeto D., Ryll T., Chamow S. and Chung J.D. 2003. Application of factorial design to accelerate identification of CHO growth factor requirements. Biotechnol. Prog. 19(1): 52–57.

Fenner F., Henderson D.A., Arita I., Jezek Z. and Ladnyi I.D. 1988. Smallpox and its Eradication. World Health Organization, Geneva.

Greenberg R.N., Kennedy J.S., Clanton D.J., Plummer E.A., Hague L., Cruz J., Ennis F.A., Blackwelder W.C. and Hopkins R.J. 2005. Evaluation of Safety and Immunogenicity of a new cell-cultured smallpox vaccine (CCSV) compared with a calf lymph derived smallpox vaccine (Dryvax®). Lancet 365: 398–409.

Henderson D.A., Inglesby T.V., Bartlett J.G., Ascher M.S., Eitzen E., Jahrling P.B., Hauer J., Layton M., McDade J., Osterholm M.T., O'Toole T., Parker G., Perl T., Russell P.K. and Tonat K. 1999. Smallpox as a biological weapon: medical and public health management. J. Am. Med. Assoc. 281: 2127–2137.

Jacobs J.P., Jones C.M. and Baille J.P. 1970. Characteristics of a human diploid cell designated MRC-5. Nature 227: 168–170.

Lambert K. and Pirt S.J. 1975. The quantitative requirements of human diploid cells (strain MRC-5) for amino acids, vitamins and serum. J. Cell Sci. 17(3): 397–411.

Litwin J. 1970. Standardization of human diploid cell cultivation. Appl. Microbiol. 20(6): 899–906.

Litwin J. 1971. Standardization of human diploid fibroblast cultivation: trypsinization procedure. Appl. Microbiol. 21(2): 169–174.

Moran E.B., McGowan S.T., McGuire J.M., Frankland J.E., Oyebade I.A., Waller W., Archer L.C., Morris L.O., Pandya J., Nathan S.R., Smith L., Cadette M.L. and Michalowski J.T. 2000. A systematic approach to the validation of process control parameters for monoclonal antibody production in fed-batch culture of a murine myeloma. Biotech. Bioeng. 69(3): 242–255.

Namba M. and Kimoto T. 1979. An attempt for improving plating efficiency of human diploid fibroblasts. Gann 70(1): 21–28.

Pani G., Colavitti R., Bedogni B., Anzevino R., Borrello S. and Galeotti T. 2000. A redox signaling mechanism for density-dependent inhibition of cell growth. J. Biol. Chem. 275(49): 38891–38899.

Randers-Eichhorn L., Bartlett R.A., Frey D.D. and Rao G. 1996. Noninvasive oxygen measurements and mass transfer considerations in tissue culture flasks. Biotech. Bioeng. 51(4): 466–478.

Ryan J.M., Sharf B.B. and Cristofalo V.J. 1975. The influence of culture medium volume on cell density and lifespan of human diploid fibroblasts. Exp. Cell Res. 91(2): 389–392.

Taylor W.G., Camalier R.F. and Sanford K.K. 1978. Density-dependent effects of oxygen on the growth of mammalian fibroblasts in culture. Cell Physiol. 95(1): 33–40.

Wieser R.J. and Oesch F. 1986. Contact inhibition of growth of human diploid fibroblasts by immobilized plasma membrane glycoproteins. Cell Biol. 103(2): 361–367.